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A novel strategy for Cr(III) and Cr(VI) analysis in dietary supplements by speciated isotope dilution mass spectrometry

Nora Unceta, Maider Astorkia, Zuriñe Abrego, Alberto Gómez-Caballero, M. Aránzazu Goicolea, Ramón J. Barrio *

Department of Analytical Chemistry, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain

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ABSTRACT

In recent years, Cr speciation in dietary supplements has become decisive in the evaluation of their health risks. Despite being an beneficial micronutrient, Cr(III) can be toxic at living organisms at high concentrations, while Cr(VI) is known to be highly toxic and carcinogenic. The main objective of this work was to optimize an analytical methodology for the extraction and accurate quantification of Cr(III) and Cr(VI) in dietary supplements. The extraction of Cr species was carried out with 50 mM EDTA solution on a hotplate under optimized conditions. Special attention was paid to bidirectional species transformations. No noticeable oxidation of Cr(III) into Cr(VI) was observed and the reduction to Cr(III) only occurred at very high Cr(VI) concentrations. Cr(III) as $Cr(EDTA)^-$ complex was chromatographically separated from Cr(VI), retained as CrO_4^{2-}, on an anion exchange column coupled to inductively coupled plasma mass spectrometry (LC-ICP-MS). The limit of quantification (0.08 $\mu g\ g^{-1}$) was below the limit established for Cr enriched yeasts by the European Union. Eleven dietary supplements were analyzed and Cr(III) and Cr(VI) quantification was carried out by external calibration monitoring ^{52}Cr isotope and by speciated isotope dilution mass spectrometry (SIDMS) adding $^{50}Cr(III)$ and $^{53}Cr(VI)$ spikes. Total Cr was also quantified by ICP-MS and mass balance between total Cr and the sum of Cr(III) and Cr(VI) was achieved. In eight of the eleven tested supplements Cr(III) calculated amounts were higher than those indicated by the manufacturer, but only one of them exceeded the 250 $\mu g\ day^{-1}$ recommended by World Health Organization (WHO). In contrast, it is worth noting that Cr(VI) amounts beyond the recommendations of the European Union for Cr enriched yeasts were found in five supplements. These results revealed that more accurate and rigorous quality assurance protocols should be applied to the testing of the final products, including the analysis of both Cr(III) and Cr(VI).

1. Introduction

Chromium (Cr) is a naturally occurring heavy metal that can exist in several oxidation states (i.e. II, III, IV, V and VI). However, only Cr (III) and Cr(VI) are stable enough in the environment. Their stability is mainly conditioned by redox potential and pH; under acidic conditions, the high redox potential of Cr(VI)/Cr(III) couple promotes the stabilization of Cr(III), whereas under alkaline conditions Cr(VI) is more stable [1,2]. These chemical properties determine their stability, bioavailability and mobility in the environment, as well as in living organisms [3].

Cr(III) is considered a beneficial micronutrient in the human diet. It promotes insulin binding to insulin-sensitive cells improving its action, and consequently, it has an impact on carbohydrate and lipid metabolisms [4, 5]. Due to these properties, Cr(III) is widely used in dietary supplements marketed to reduce fat mass and increase lean body mass [6]. However, there are evidences that show that even being beneficial, Cr(III) can be toxic to living organisms at high concentrations. Some studies have concluded that Cr(III) causes oxidative DNA damage and mutagenic damage in cell culture [7,8], as well as sterility, lethal mutations and cancer in experimentation animals [9,10]. Even if these effects are unlikely to occur in humans exposed to nutritional supplement levels of Cr(III), excessive intake of Cr(III) supplements is not warranted at this time. To date, there is not any regulation available about the concentration of Cr(III) in foods or dietary supplements. Some organizations and expert groups have suggested different values for the daily intake, being the most restrictive one the recommendation of the World Health Organization (WHO) that considers that the upper limit of safe range of population mean intakes should not exceed 250 $\mu g\ day^{-1}$ [11].

* Corresponding author.
E-mail address: r.barrio@ehu.es (R.J. Barrio).

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Cr(VI), in contrast, is regulated by the Dangerous Substances Directive (67/548/EEC) for being highly toxic [12]. A continuous oral exposure may cause damage to the respiratory tract, liver, kidneys and to the gastrointestinal and immune system as well as cause dermatitis and skin ulcers due to a constant contact [13]. Furthermore, Cr(VI) is classified as highly toxic species and carcinogenic to human beings by EPA and as Class I carcinogenic species by the International Agency for Research on Cancer (IARC) [14,15].

Despite being primarily added in dietary supplements as Cr(III) picolinate, nicotinate or chloride, the oxidation of Cr(III) to Cr(VI) can occur due to critical steps during formulation involving the variation of the pH or redox potential or to the presence of additional oxidizing compounds in the formulation [16]. Unreliable sources of raw materials may also contribute to Cr(VI) content. Consequently, total Cr quantification provides no real information about the health risks of these supplements, and the quantification of both Cr(III) and Cr(VI) in the final product becomes beneficial.

To date, chromium analysis in dietary supplements has been mostly focused on the analysis of total Cr [17–21]. To our knowledge, only three procedures have been reported regarding Cr(III) and Cr(VI) analysis in yeast [22] or in dietary supplements [16,23]. For the determination of Cr species in Cr enriched yeast, samples were digested in a 0.5 M NaOH-0.28 M Na_2CO_3 alkaline solution on a hotplate at 95 ° C for 6 h and Cr(III) and Cr(VI) were analyzed by anion-exchange liquid chromatography coupled to inductively coupled plasma mass spectrometry (LC-ICP-MS) [22]. With this procedure, significant bidirectional species transformation occurred and in order to correct these interconversions species-specific double-spike isotope dilution was required. The analysis of Cr(III) and Cr(VI) in dietary supplements was also carried out in strong basic conditions by applying EPA Method 3060 [24], proposed for the extraction of both soluble and insoluble Cr(VI) from soil, sludge, sediment and similar [16]. This method consists in a digestion with a 0.5 M NaOH-0.28 M Na_2CO_3 solution, 0.5 M K_2HPO_4–0.5 M KH_2PO_4 buffer and $MgCl_2$ heating at 90-100 ° C for 60 min. While Cr(VI) was extracted from the sample in the alkaline solution and quantified by LC-ICP-MS, Cr(III) formed a precipitate that had to be digested and quantified by ICP-MS. Speciated isotope dilution mass spectrometry (SIDMS) and isotope dilution mass spectrometry (IDMS) with mass balance were also used in order to correct interconversions between species.

Consequently, it was considered necessary the development of a simple sample processing protocol for the extraction of Cr(III) and Cr (VI) from dietary supplements as an alternative to the treatment with $NaOH/Na_2CO_3$ that would minimize species interconversions and ease both sample treatment and quantification protocol. Previous works in environmental samples proposed the addition of Cr(III) complexing agents during Cr(VI) extraction. Thus, the addition of ethylenediaminetetraacetic acid (EDTA) [25–27], the ethylenediamine-N, N′-disuccinic acid (EDDS) [28], 2,6-pyridinedicarboxylic acid (PDCA) [29] or diethylenetriaminepentaacetic acid (DTPA) [30,31] has been reported to improve the efficiency of Cr(VI) extraction and the stabilization of the extracted Cr compounds [27]. Some studies recommend the use of EDTA as extracting solution in Cr speciation since the formation of $Cr(EDTA)^-$ complex prevents Cr(III) oxidation to Cr(VI) and enables its separation from Cr(VI) by anionic chromatography [27,32]. The goal of this research was therefore to evaluate EDTA as extractant for the speciation of Cr(III) and Cr(VI) in dietary supplements by anion-exchange LC-ICP-MS. Cr(III) complexing reactions are known to be slow and, in order to accelerate this process, incubation on a hotplate and microwave assisted extraction (MAE) were evaluated. Special attention was paid to interconversion reactions that could occur in the extraction procedure by adding isotopically enriched ^{50}Cr(III) and ^{53}Cr(VI).

2. Material and methods

2.1. Reagents and materials

A multi-elemental stock standard solution containing 10 μ g mL^{-1} Cr in 5% HNO_3 (Inorganic Ventures, Christiansburg, VA, USA) was used for preparing calibration standards for total Cr quantification. Natural abundance stock standard solutions of 100 μg mL^{-1} Cr(III) in 2% HNO3 (VHG, Manchester, NH, USA) and 1000 μg mL^{-1} Cr(VI) in water (LGC standards, Manchester, NH, USA) were used for speciation analysis by external calibration. Isotopically enriched stock standard solutions (or spikes) of ^{50}Cr(III) in 2% HNO3 (^{50}Cr (77.17%), ^{52}Cr (21.53%), ^{53}Cr(0.94%), ^{54}Cr(0.36%)) and ^{53}Cr(VI) in water (^{50}Cr (0.08%), ^{52}Cr (2.910%), ^{53}Cr(97.010%), ^{54}Cr(< 0.001%)) were supplied by ISC Science (Oviedo, Spain), and their isotopic abundances and concentration were determined against a natural abundance Cr standard solution by reverse isotope dilution analysis as follows: a known amount of the isotopic enriched standard was mixed with a known amount of the natural isotopic abundance standard and the altered isotope ratio, measured by ICP-MS, was used in the calculation of the concentration of the isotopically enriched standard in the same way as in any conventional isotope dilution assay, with the peculiarity that in this case the species to determine were the enriched ones. ^{50}Cr(VI) was not detected in the ^{50}Cr(III) tracer neither ^{53}Cr(III) in the ^{53}Cr(VI) tracer. Table 1 shows the isotopic abundances and concentrations of each spike.

Tetrasodium ethylenediaminetetraacetate (EDTA) (purity > 97%) used as extracting agent as well as for the mobile phase was supplied by Sigma-Aldrich (St. Louis, USA). $Ca(NO_3)_2 \cdot 4H_2O$ and $MgCl_2 \cdot 6H_2O$ used in the optimization of EDTA concentration were supplied by Panreac (Barcelona, Spain). The 65% HNO_3 used for acid digestion of the dietary supplements was in-house purified by sub-boiling distillation in a Teflon™ still (Savillex Corp., Minnetonka, Minnesota, USA). All solutions and the mobile phase were prepared in ultra-high purity water obtained from tap water pre-treated by Elix reverse osmosis and subsequent filtration by a Milli-Q system from Millipore (Bedford, MA, USA). The mobile phase was filtered prior to use through 0.1 μm filters from Millipore Omnipore (Watford, Ireland).

2.2. Instrumentation

All the analysis were carried out in a 7500ce ICP-MS model from Agilent Technologies (Palo Alto, CA, USA), equipped with a MicroMist micro-uptake glass concentric nebulizer (Glass Expansion, West Melbourne, Victoria, Australia). To remove polyatomic interferences that could affect Cr isotopes, the Agilent Octopole Reaction System (ORS) was used in He collision mode. ICP-MS working conditions were optimized each time with a standard tuning solution containing ^{7}Li, ^{24}Mg, ^{59}Co, ^{89}Y, ^{140}Ce and ^{205}Tl in 2% HNO_3 in order to increase sensitivity and reduce oxides and

Table 1
Isotopic abundances and concentrations of the isotopically enriched ^{50}Cr(III) and ^{53}Cr(VI) solutions employed as spike.

	Concentration[a] (μg g^{-1})	**Isotope**	**Abundance**[a] (%)
^{50}Cr(III)	103.5 ± 1.1	50	76.9 ± 1.4
		52	21.7 ± 1.2
		53	1.0 ± 0.1
		54	0.34 ± 0.05
^{53}Cr(VI)	126.2 ± 0.7	50	0.07 ± 0.04
		52	2.9 ± 0.5
		53	96.8 ± 0.6
		54	0.22 ± 0.05

[a] The uncertainty is expressed as the standard deviation of n=3 replicates.

doubly charged ions formation that could interfere in the measurement.

Speciation analysis was accomplished on an Agilent 1100 Series HPLC system fitted with a Rheodyne 7725i model injection valve and a 100 μL sample loop. Chromatographic separation was carried out on a Metrosep A Supp 5 250 (PEEK, 250 × 4.0 mm, 5 μm) anion exchange column equipped with a Metrosep A Supp 4/5 Guard (PEEK, 5 × 4.0 mm) guard column both from Metrohm (Herisau, Switzerland). 2 mM EDTA solution at pH 11 was used as the mobile phase at a flow rate of 0.7 mL min^{-1}. This mobile phase provided a suitable chromatographic separation of Cr(III) and Cr(VI) in less than 8 min. The column was directly connected to the nebulizer by means of a 22 cm length and 0.25 mm diameter PEEK capillary. Once in the ICP-MS, *m/z* 50, 52 and 53 corresponding to the main Cr naturally occurring isotopes were monitored. The chromatograms were processed using the Agilent Technologies ICP-MS Plasma Chromatographic System software. Table S1 (Supplementary material) summarizes chromatographic separation conditions as well as the ICP-MS working conditions.

Acid digestion of the supplements for total Cr quantification was carried out in a Speedwave Four model microwave oven of Berghof (Eningen, Germany), equipped with DAP-60 Teflon vessels, and controlled by a Power PC 5200 software. Samples and standards were weighted in Teflon™ microcapsules which were subsequently introduced in the corresponding sample vessels.

For speciation analysis, the optimization of the sample treatment step was carried out both in a microwave oven model MARSXpress from CEM (Matthews, NC, USA) and on a stirring hotplate model C-Mag HS7 from IKA (Staufen, Germany). The samples, once processed, were centrifuged in an Allegra X-22R centrifuge from Beckman Coulter (Pasadena, CA, USA). All the solutions were prepared by weighing in an analytical balance model ME235P of Sartorius (Bradford, MS, USA).

2.3. Real samples

Eleven (11) chromium dietary supplements comprising tablets, capsules and liquid mixture of different commercial brands were purchased from the internet. Containers were labeled with codes and stored at room temperature in a clean area until processing.

2.4. Sample preparation procedures

2.4.1. Total Cr analysis by ICP-MS

For total Cr analysis, solution mode analysis was performed by ICP-MS after microwave assisted acid digestion of the supplements. All the samples were homogenized previous to sample preparation. 0.1 g of sample was weighed and transferred to a Teflon vessel and 7 mL of 65% HNO_3 purified by sub-boiling distillation were added. The vessels were closed and heated by a one-step MW program at 180 °C for 10 min and 80% power. After cooling, ultra-high purity water was added until 15 g and then diluted 1:100 for subsequent measurement by ICP-MS. Total Cr quantification was carried out by external calibration and isotope dilution mass spectrometry (IDMS).

2.4.2. Cr(III) and Cr(VI) analysis by LC-ICP-MS

Cr(III) and Cr(VI) speciation analysis was performed adding to 0.1 g of dietary supplement 4 g of an 50 mM EDTA solution (pH 11). The mixture was heated on a hotplate at 95 °C for 60 min under stirring. The achieved extract was then centrifuged at 8000 rpm, avoiding sample filtration and therefore the risk of contamination or even interconversions. Finally, the supernatant was diluted 1:5. Likewise, Cr(III) and Cr(VI) quantification was performed by external calibration and speciated isotope dilution mass spectrometry (SIDMS).

3. Results and discussion

3.1. Total Cr analysis by ICP-MS

For total Cr quantification, all dietary supplements were digested in acidic media in a microwave oven following the procedure reported in Section 2.4.1. All of them were digested in three replicates and each replicate was analyzed three times. Initially, the concentration of total Cr was determined by external calibration. Calibration standards (n=8) were prepared by doping a reagent blank obtained after the microwave digestion and dilution of 7 mL of sub-boiling 65% HNO_3 with 10 μg mL^{-1} Cr stock standard solution to yield concentrations ranging from 5 to 200 ng g^{-1} (r=0.996). The concentration value obtained for each supplement was converted to total Cr per dose (Table 2). However, except for A and E supplements ($p > 0.05$), the difference between the calculated amount and the values reported by the manufacturer were statistically significant according to Student's *t*-test at significance levels shown in Table 2. Consequently, so as to verify these results, the quantification of total Cr by isotope dilution mass spectrometry (IDMS) was considered necessary.

For IDMS, a suitable amount of enriched ^{53}Cr spike was added to each sample to result in a ratio of concentrations between analyte and spike in the range of 0.1–10. According to US EPA Method 6800, ideal isotope ratio ($^{53}Cr/^{52}Cr$) is 1:1. Therefore, for each dietary supplement sample, the amount of ^{53}Cr spike added was calculated taking into consideration the amount of ^{52}Cr expected in 0.1 g of sample, obtaining a $^{53}Cr/^{52}Cr$ close to 1:1.

Subsequently, the mixture was subjected to the microwave acid digestion according to the procedure described previously, to finally measure the isotopic ratios by ICP-MS. Mass bias correction factors were determined by analyzing a 100 ng g^{-1} natural abundance Cr standard solution by ICP-MS at the beginning and at the end of the analysis sequence of each supplement. Total Cr in dietary supplements was calculated by measuring 52/53 isotope ratio of the mixture, together with the known isotopic abundance of both spike and sample and the known amount of the sample and the spike, and following the equations for total Cr by IDMS reported in EPA Method 6800 [33]. Likewise, the results obtained by IDMS were also statistically different from the values reported by the manufacturer, except for A and E supplements which were undistinguishable ($p > 0.05$) (Table 2). However, when comparing the values for total Cr obtained by external calibration and IDMS, the Student's test confirmed that there were not statistically significant differences at $p > 0.05$, demonstrating the validity of the results.

Table 2
Total Cr amount per dose in dietary supplements by external calibration and IDMS. Statistical comparison by Student's *t*-test of the values reported by the manufacturer and the calculated amounts for each supplement.

SAMPLE	Cr (μg/dose)[a]	Cr (μg/dose) External calibration[b]	Cr (μg/dose) IDMS[b]
A (tablet)	240	237 ± 7	242 ± 11
B (tablet)	200	230 ± 7*	248 ± 3**
C (tablet)	200	212 ± 4*	217 ± 3*
D (tablet)	200	235 ± 8*	237 ± 9*
E (capsule)	200	202 ± 5	214 ± 9
F (capsule)	200	230 ± 5**	211 ± 3*
G (capsule)	200	246 ± 3**	249 ± 2**
H (liquid capsule)	200	145 ± 3**	150 ± 1**
I (tablet)	500	605 ± 6**	609 ± 19**
J (capsule)	20	30 ± 2**	38 ± 3*
K (capsule)	98	220 ± 11**	194 ± 6**

p value is expressed as *$p < 0.05$,**$p < 0.01$,***$p < 0.001$

[a] Values reported by the manufacturer.

[b] The uncertainty is expressed as the standard deviation of n=3 replicates.

3.2. Cr (III) and Cr (VI) analysis by LC-ICP-MS

3.2.1. Chromatographic separation

In aqueous solution, Cr(III) tends to form cationic complexes ($[Cr(H_2O)_6]^{3+}$) and hydrolysis products ($Cr(OH)^{2+}$or $Cr(OH)_2{}^{+}$), while Cr(VI) forms anionic compounds as hydrogen chromate ($HCrO_4{}^{-}$) and chromate ($CrO_4{}^{2-}$) [2]. Metrosep A Supp 5 250 is an anion exchange column, thus, only Cr(VI) as $CrO_4{}^{2-}$ is retained on it, and the formation of a negative Cr(EDTA)$^-$ complex is required for the retention of Cr(III). In order to guarantee the stability of the Cr(EDTA)$^-$ complex, the use of a mobile phase with EDTA is recommended. However, the use of organic compounds in the mobile phase implies an increase of polyatomic interferences that may affect the most abundant ^{52}Cr and ^{53}Cr isotopes detection: $^{35}Cl^{16}OH^+$, $^{40}Ar^{12}C^+$and $^{37}Cl^{14}NH^+$interfere with ^{52}Cr and $^{40}Ar^{13}C^+$, $^{40}Ar^{12}CH^+$and $^{37}Cl^{16}O^+$with ^{53}Cr[34]. Therefore, and in order to eliminate as much polyatomic interferences as possible, a mobile phase with 2 mM EDTA, at a flow rate of 0.7 mL min^{-1}, was employed together with an Octopole Reaction System (ORS) in collision mode with He as cell gas at 4 mL min^{-1} flow [35]. Under these conditions, a suitable chromatographic separation of Cr(III) and Cr(VI) was achieved in less than 8 min.

As shown in Fig. 1, the peak at 5 min corresponds to Cr(III) as Cr(EDTA)$^-$ complex and the peak at 7.4 min to Cr(VI) ion as $CrO_4{}^{2-}$. Their isotopic abundances correspond with Cr natural isotopes (^{50}Cr 4.3%, ^{52}Cr 83.8%, ^{53}Cr 9.5%).

3.2.2. Sample processing

As previously reported, using EDTA as extractant in Cr speciation allows the formation of Cr(EDTA)$^-$ complex, thereby preventing Cr(III) oxidation to Cr(VI) and allowing its separation from Cr(VI) by anionic chromatography. The formation of Cr(EDTA)$^-$ complex is known to be slow and it is usually carried out on a hotplate [32], or even accelerated in a microwave oven [27,36]. In this work, both possibilities were evaluated in order to achieve a good extraction efficiency avoiding interconversion reactions.

For the optimization of Cr(EDTA)$^-$ complex formation, different EDTA concentrations, pH values, reaction times and incubation temperatures were tested. Due to the difficulty in obtaining blank matrices free of Cr, the optimization of these parameters was performed employing the supplement labeled as A for which calculated total Cr amount was consistent with the values reported by the manufacturer. For preliminary studies, to 0.1 g of A supplement 5 g of an EDTA solution were added and the mixture was heated on a hotplate at 95 °C for 60 min while stirring. After that, the extract was centrifuged at 8000 rpm and the supernatant thus obtained was diluted 1:5 in ultra-high purity water. The influence of EDTA concentration on Cr(III) signal was tested in the range of 10–200 mM. A slight enhancement of the area of the chromatographic peak corresponding to Cr(EDTA)$^-$ was observed till 50 mM EDTA. Nevertheless, the presence of divalent cations such as calcium and magnesium in multicomponent dietary supplements may affect the formation of Cr(EDTA)$^-$ complex by competition. So, this time, to 0.1 g of the supplement 5 g of EDTA solution and 1 g of 200 μg g^{-1} Ca and Mg solution were added as well, taking into account the highest amount of these divalent cations reported in supplements with Cr. The experimental results revealed that at these EDTA concentration levels Ca and Mg do not compete with Cr(III) (Fig. 2A). Subsequently, and with the aim of improving sensitivity, the amount of 50 mM EDTA required was optimized in the range of 1 and 5 g. The results showed that the recovery of Cr increased with the volume of EDTA till 4 g and then reached saturation.

As previously mentioned, the pH of the extraction solution is also a crucial parameter considering that it determines the stability of Cr species. Because of the low stability of Cr(VI) in acidic media, neutral and basic media were assayed. The peak area corresponding to Cr(EDTA)$^-$ complex did not change at pH values between 7 and 12, so it was decided to work at pH 11 as this was the value of the 50 mM EDTA solution itself. Subsequently, the influence of incubation time was studied at 15, 30, 45, 60 and 75 min. Significant variation of the peak area of Cr(III) was observed showing that complexation was not complete till 45 min (Fig. 2B). Nevertheless, and in order to guarantee the total extraction of Cr(III) in supplements, an incubation time of 60 min was chosen. Finally, the influence of temperature on the formation of Cr(EDTA)$^-$ complex was studied at 75 and 95 °C, since higher temperatures have been reported to increase the reduction of Cr

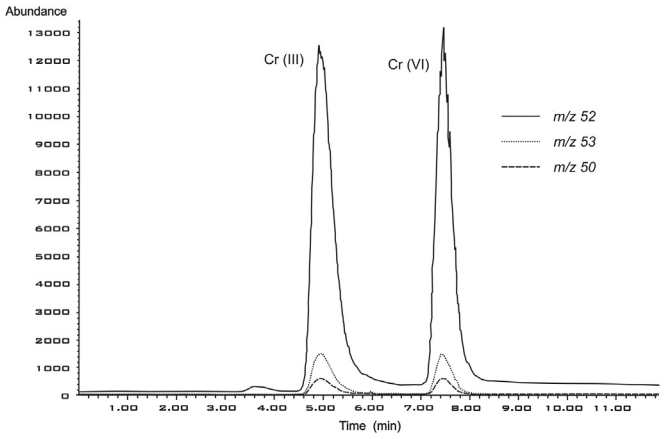

Fig. 1. Chromatogram corresponding to 40 ng g^{-1} of Cr(III) and Cr(VI), once extracted with 50 mM EDTA, using as mobile phase 2 mM EDTA (pH=11) at 0.7 mL min^{-1}.

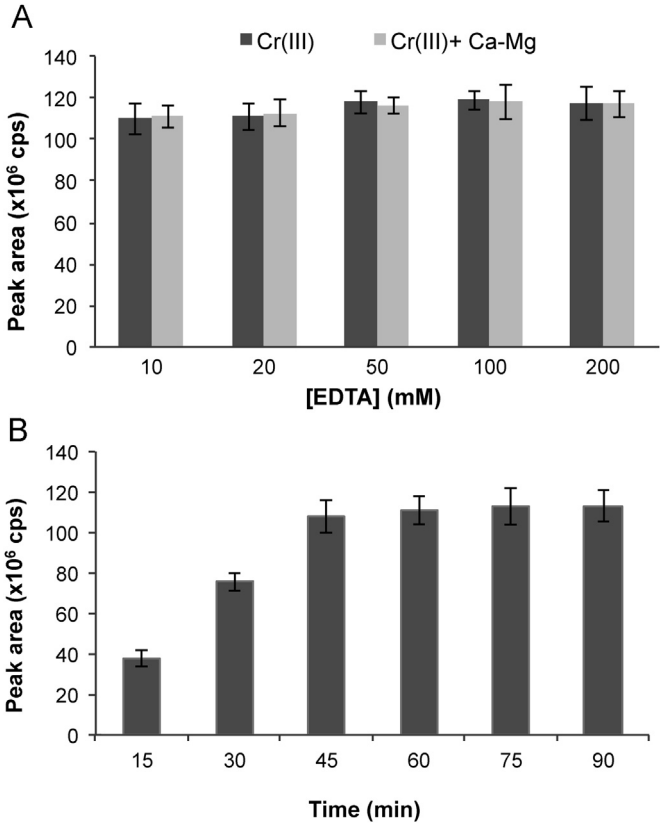

Fig. 2. Effect of (A) EDTA concentration in absence and presence of Ca and Mg and (B) incubation time on Cr(EDTA)$^-$ complex formation for A dietary supplement incubated with 50 mM EDTA at 95 °C.

(VI) to Cr(III) [24,27]. As expected, no variation in Cr(III) response was observed and 95 °C was chosen to ensure the extraction of Cr (VI).

In order to obtain faster complexation kinetics and thus reduce the analysis time, microwave assisted extraction (MAE) was evaluated. The analysis conditions were the same as for the hotplate (0.1 g sample, 4 g of 50 mM EDTA at pH 11). Initially, a power limit of 800 W and a ramp time of 5 min to reach 95 °C were applied, which was maintained for 10 min while stirring. After centrifuging at 8000 rpm, the supernatant was diluted 1:5 with ultra-high purity water. Under these conditions, the recovery of Cr(III) ranged between 89.8 and 95.1%.

Comparing the chromatograms of A supplement, when it was processed on the hotplate both Cr(III) and Cr(VI) were observed, while no peak for Cr(VI) was detected in the extract obtained after MAE (Fig. 3). Two hypothesis were considered: (i) There was not Cr (VI) in the sample, thus its presence was due to the oxidation of Cr (III) under hotplate extraction conditions; (ii) There was Cr(VI) in the sample but under MAE it was reduced to Cr(III).

To clarify this fact it was necessary to study the interconversion reactions between Cr(III) and Cr(VI) on a hotplate and in a microwave oven. For this, some authors calculated reduction and oxidation factors using double spike SIDMS applying the mathematical approach based on isotope pattern deconvolution [27]. However, in order to simplify the procedure this study was performed as follows: First, A supplement, unspiked and spiked with 50, 100 and 200 $\mu g\ g^{-1}$ of $^{50}Cr(III)$, was processed on the hotplate under the optimized conditions. The isotopically enriched spike was added after the addition of the EDTA solution so as to avoid the interconversions of the species due to the matrix. As shown in Fig. 4A, while the intensity of the peak corresponding to $^{50}Cr(III)$ increased when increasing its concentration, the signal corresponding to $^{50}Cr(VI)$ did not vary. This demonstrated that no oxidation of Cr(III) to Cr(VI) occurred on the hotplate and confirms that under these conditions $Cr(EDTA)^-$ complex prevents the oxidation of Cr(III).

Similarly, and in order to check whether a reduction of Cr(VI) would occur under hotplate conditions, A supplement unspiked and spiked with $^{53}Cr(VI)$ to yield 50, 100 and 200 $\mu g\ g^{-1}$ was also processed. When 50 $\mu g\ g^{-1}$ of $^{53}Cr(VI)$ were added, no increase of $^{53}Cr(III)$ signal was observed comparing to the unspiked sample. In contrast to this, $^{53}Cr(III)$ signal increased 7% and 36% in the samples spiked with 100 and 200 $\mu g\ g^{-1}$ respectively, as a consequence of the reduction of $^{53}Cr(VI)$ (Fig. 4B). Considering that the amount of Cr(VI) expected in the supplements is lower than 50 $\mu g\ g^{-1}$, its reduction could be considered negligible.

Finally, and assuming the stability of $Cr(EDTA)^-$ complex, the reduction of Cr(VI) to Cr(III) was assessed under MAE conditions. In the same way, A supplement was analyzed unspiked and spiked with 50, 100 and 200 $\mu g\ g^{-1}$ of $^{53}Cr(VI)$. As expected, the signal of

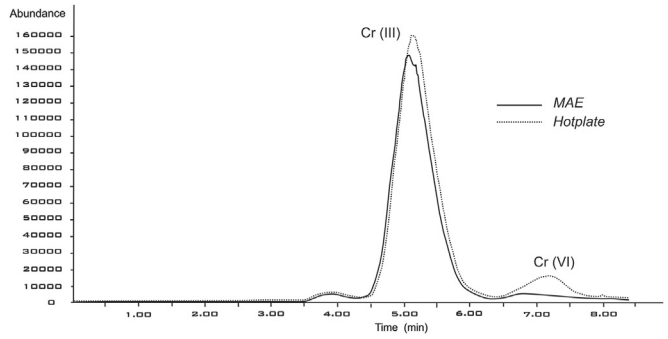

Fig. 3. Chromatograms obtained (*m/z* 52) after processing A supplement on a hotplate and by MAE.

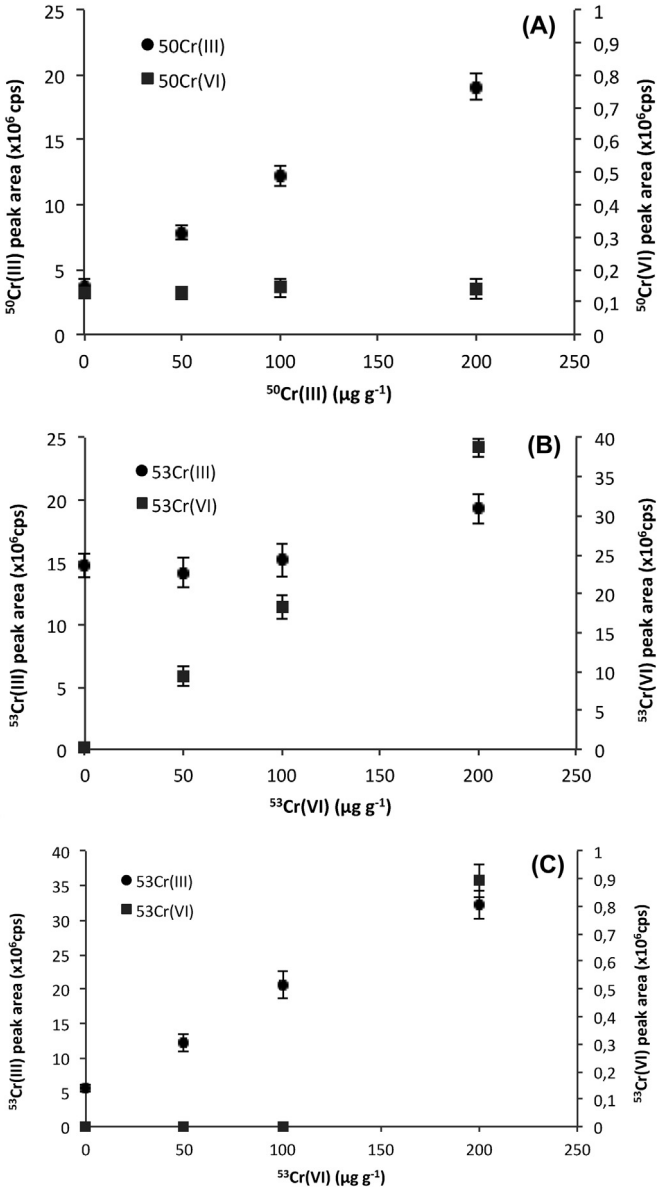

Fig. 4. Cr(III) and Cr(VI) peak areas after processing on a hotplate when (A) $^{50}Cr(III)$ and (B) $^{53}Cr(VI)$ were added and (C) by MAE when $^{53}Cr(VI)$ was added.

$^{53}Cr(III)$ increased proportionally to the concentration of $^{53}Cr(VI)$ added while the signal of $^{53}Cr(VI)$ was barely detected at higher concentrations demonstrating that its reduction was almost complete (Fig. 4C).

Several attempts were carried out in order to avoid Cr(VI) reduction when MAE was used. This way, the power limit was lowered to 400 W, the minimum value allowed by the microwave oven used in this study, and the extraction time reduced to 5 min. A procedure based on two cycles was also assayed as reported by Fabregat et al. [27]: a ramp time of 1 min followed by 5 min at 90 °C and, after cooling to room temperature, a second cycle of a 1 min ramp and 5 min at 110°. Under all tested conditions, the reduction of Cr(VI) was unavoidable, leading to the conclusion that the extraction on a hotplate, despite the longer analysis time, was the most suitable procedure for the extraction of Cr(III) and Cr(VI) from dietary supplements.

3.2.3. Method validation

Validation of the method was performed under the optimal hotplate extraction conditions reported in Section 2.4.2. Due to the

different composition of the tested supplements, it was not possible to obtain a blank matrix free of Cr. The large volume of EDTA used in the sample treatment and the subsequent dilution of the supernatant made it guess that the matrix effect would be insignificant. In order to confirm whether the matrix effect was negligible or not, ^{50}Cr(III) and ^{53}Cr(VI) were added to previously extracted and diluted A supplement. The matrix effect, calculated as the ratio of the peak area increase in the matrix to the peak area in a working solution, was 99.8 ± 4.7% and 98.4 ± 4.5% for ^{50}Cr(III) and ^{53}Cr(VI) respectively. According to these values, it was stated that matrix effect was not significant and therefore, that external calibration in working standard solutions would be suitable for the quantification of Cr(III) and Cr(VI) in dietary supplements following the developed procedure.

Calibration standards were prepared by adding to 4 g of 50 mM EDTA a suitable amount of Cr(III) and Cr(VI) to yield, once extracted and diluted 1:5, concentrations ranging between 0.4 and 5000 ng g^{-1} (n=12) and 0.4 and 100 ng g^{-1} (n=8) respectively. These standards underwent the optimized sample treatment procedure. Linear regression equations were obtained by plotting the peak area versus the analyte concentration, and correlation coefficients were 0.9991 and 0.9996 for Cr(III) and Cr(VI) respectively. The limit of quantification, defined as the minimum concentration quantified with an accuracy of 100 ± 20%, was 0.4 ng g^{-1} for both Cr(III) and Cr(VI), and corresponds to 0.08 μg g^{-1} in sample. This value is low enough to make it well suited for the quantification of Cr(VI) levels beyond the limit established by the Commission Regulation (EU) No 119/2014 [37].

The method accuracy was evaluated in terms of trueness and precision. Under the established conditions, the trueness of the measurements was assessed through recovery of ^{52}Cr(III) and ^{53}Cr(VI) from A supplement. In absence of a reference material and blank matrices free of Cr, the recovery of Cr(III) was calculated considering that total Cr in the sample was as Cr(III). It was not feasible to add isotopically labeled ^{50}Cr(III) to the supplement because ^{50}Cr(III) isotope from natural ^{52}Cr(III) of the sample was not negligible. Although small amounts of Cr(VI) were detected in A supplement, ^{53}Cr(VI) was negligible, and the recovery of Cr(VI) was calculated by doping A supplement with 0.02 g of ^{53}Cr(VI) 5 μg g^{-1}. The recoveries were found to be in the range of 95.3–104.9% and 94.4–104.8% for ^{52}Cr(III) and ^{53}Cr(VI) respectively. As expected, EDTA was demonstrated to be effective not only for Cr(III) complexation, but also for Cr(VI) extraction. The precision of the proposed method was calculated carrying out five determinations of A supplement doped with isotopically labeled ^{53}Cr(VI) that were repeated for 3 days. The RSD of the repeatability was below 4.7% and 4.6% for Cr(III) and Cr(VI) respectively, whereas intermediate precision did not exceed 12.0% and 10.1%.

3.2.4. Cr(III) and Cr(VI) quantification in real samples

In order to verify the reliability of the developed method, Cr(III) and Cr(VI) were quantified in eleven dietary supplements by both external calibration and speciated isotope dilution mass spectrometry (SIDMS). Quantification by external calibration was performed preparing a series of standard solutions as indicated in Section 3.2.3. SIDMS was carried out by adding weighed amounts of the isotopically enriched ^{50}Cr(III) and ^{53}Cr(VI) after mixing the sample with 50 mM EDTA, so that the ratio between the amount of analyte and spike just before the sample treatment did not exceed 1:10 or 10:1. Samples were extracted in three replicates that were analyzed three times. To calculate the concentration of Cr species, the mass bias corrected 52/50 and 52/53 isotope ratios were considered for Cr(III) and Cr(VI) peaks respectively.

According to EPA Method 6800, SIDMS should be used for the quantification of elemental species. SIDMS assumes that all the converted species can be found in other species that are monitored. If only one species is of interest, such as Cr(VI), the sample could be single spiked with ^{53}Cr(VI) based on the assumption that only unidirectional conversion, the reduction of Cr (VI) to Cr(III), can occur after spiking. In this case, the equation used is similar to the one used in the determination of total Cr. If Cr(III) and Cr(VI) species are of interest, the sample should be double spiked with ^{50}Cr(III) and ^{53}Cr(VI), and both species concentrations and species conversions are mathematically deconvoluted on the basis of a matrices series resolution.

According to the study on interconversion reactions in Section 3.2.2, the complexation of Cr(III) with EDTA avoids its oxidation to Cr(VI) and the reduction of Cr(VI) that may occur in dietary supplements is negligible under the optimized conditions. Therefore, it can be assumed that interconversions are not significant and Cr (III) and Cr(VI) can be considered as independent species. This allows to calculate Cr(III) and Cr(VI) concentrations using for each of them the equations used for single-spike samples which are similar to that used in the determination of total Cr [33].

Cr(III) and Cr(VI) amounts expressed as μg/dose calculated after quantification by external calibration and SIDMS are shown in Table 3. For each supplement, Student's *t*-test was applied to study the similarity between the results obtained by both quantification methods. According to this test, no statistically differences were observed for the analyzed dietary supplements ($p > 0.05$) except for J and K supplements, for which calculated Cr(III) amounts were statistically different at $p < 0.05$ and $p < 0.01$ respectively. Regarding Cr(VI), it was detected in seven of the analyzed supplements but only was feasible its quantification in five of them. For these supplements, Cr(VI) amounts calculated by both quantification methods were statistically indistinguishable at $p > 0.05$.

The validity of the results was verified by mass balance between total Cr calculated by IDMS and the sum of Cr(III) and Cr(VI) obtained by SIDMS and external calibration. As shown in Fig. 5, regarding SIDMS, no statistically significant differences were observed for any of the analyzed supplements ($p > 0.05$) when comparing to total Cr. Similar results were observed when the amounts obtained after external calibration and total Cr were compared with the exception of J and K supplements, for which a significant difference was also observed at $p < 0.05$ and $p < 0.01$ respectively. It is worth noting that J and K are multicomponent dietary supplements that contain Cr(III) as well as vitamins and plant extracts, and therefore, they are not specifically marketed as Cr(III) supplements. The results suggested that this complexity in the composition implied an increased matrix effect that was not

Table 3
Cr (III) and Cr (VI) amounts in dietary supplements after quantification by external calibration and by SIDMS. Statistical comparison by Student's *t*-test of the amounts calculated by external calibration and SIDMS for each Cr specie and each supplement.

Sample	External calibration[a]		SIDMS[a]	
	Cr (III) (μg/dose)	Cr (VI) (μg/dose)	Cr (III) (μg/dose)	Cr (VI) (μg/dose)
A	243 ± 14	7.0 ± 0.7	236 ± 7	7.9 ± 0.3
B	225 ± 10	< LOQ	238 ± 8	< LOQ
C	189 ± 20	n.d.	218 ± 23	n.d.
D	225 ± 5	2.1 ± 0.2	242 ± 23	2.5 ± 0.3
E	208 ± 12	4.4 ± 0.6	215 ± 21	5.1 ± 0.1
F	227 ± 16	10.9 ± 0.8	216 ± 14	10 ± 2
G	258 ± 12	< LOQ	249 ± 27	< LOQ
H	153 ± 19	n.d.	150 ± 9	n.d.
I	603 ± 8	n.d.	603 ± 8	n.d.
J	24 ± 2	n.d.	32 ± 5*	n.d.
K	37 ± 1	5 ± 1	202 ± 12**	5.5 ± 0.9

p value is expressed as *$p < 0.05$,**$p < 0.01$,***$p < 0.001$)

[a] The uncertainty is expressed as the standard deviation of n=3 replicates.

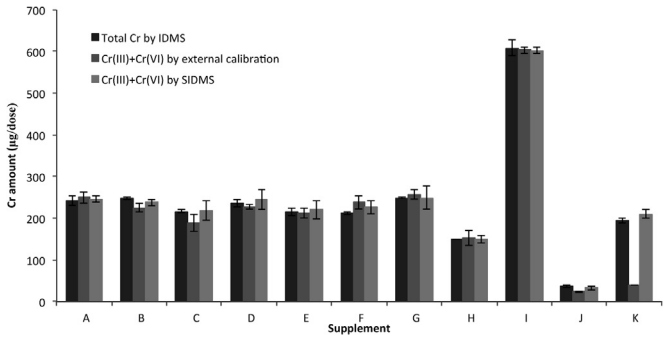

Fig. 5. Mass balance between total Cr and the sum of Cr(III) and Cr(VI) obtained by SIDMS and external calibration.

corrected by external calibration, which was prepared in an EDTA solution, while SIDMS protocol was more accurate for this kind of samples.

Despite being in some supplements higher than the values reported by the manufacturer, only I supplement exceed the 250 μg Cr(III) day^{-1} dosage established by WHO, considering that for all of them the dosage is one dose per day. To date, there is no legislation that regulates Cr(VI) daily intake in food. However, the Commission Regulation (EU) No. 119/2014 amending Directive 2002/46/EC and Regulation (EC) No 1925/2006 regarding chromium enriched yeast used for the manufacture of food supplements and Cr(III) lactate tri-hydrate added to foods, establishes that in chromium enriched yeast, as a source of chromium and containing 230–300 mg Cr kg^{-1} in the dried form as marketed, the content of Cr(VI) shall not exceed 0.2% of total chromium [37]. This means that the amount of Cr(VI) should not exceed 0.46 μg g^{-1}. In five of the analyzed supplements, Cr(VI) ranged between 2.1 and 10.9 μg g^{-1} (0.9% and 4.6% of total Cr respectively), exceeding the limit established by this Regulation. Consequently, a review of raw materials, formulation ingredients and/or production steps should be performed in order to prevent the presence of Cr(VI) in these dietary supplements. Nevertheless, it should be noted that the limit established by this regulation is proposed for yeast used for the manufacture of food supplements, and not for the food supplements themselves, and new regulations limiting not only the presence of Cr(VI) but also of Cr(III) in dietary supplements should be promoted.

4. Conclusions

An accurate method based on the extraction of Cr species from dietary supplements was developed as an alternative to strong basic extraction with NaOH-Na_2CO_3. Using EDTA as extracting agent and incubating on a hotplate under optimized conditions enables the stabilization of Cr(III) as $Cr(EDTA)^-$ complex as well as the extraction of Cr(VI). It is well known that bidirectional interconversions between Cr(III) and Cr(VI) can occur at any analysis stage, making it difficult to obtain accurate results. After a comparative study about the extraction on a hotplate and in MAE, we found that the latter favours Cr(VI) reduction to Cr(III). However, no noticeable interconversion of Cr(III) into Cr(VI) was observed under hotplate extraction conditions and the reduction to Cr(III) was only observed at very high concentrations of Cr(VI), which are not expected in dietary supplements.

The application of this procedure for the determination of Cr (III) and Cr(VI) in dietary supplements demonstrated its effectiveness. The quantification was carried out by external calibration and SIDMS and the validity of the results was verified by mass balance taking into account total Cr values. Quantification by external calibration provided accurate values for those supplements specifically marketed as Cr(III) supplements, reducing the cost of the analysis. However, SIDMS seems to be a more accurate quantification method whatever the composition of the supplement is. The limit of quantification for Cr(VI) was sufficiently low to make it well suited for the quantification of Cr(VI) levels beyond the limit established by the Commission Regulation (EU) No. 119/2014.

To sum up, the method developed is suitable for routine analysis of the more and more popular Cr dietary supplements since it allows to obtain accurate and precise information about the real health risks not only of small amounts of Cr(VI), but also of high levels of Cr(III).

Compliance with ethical standards

Conflict of interest.

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2016.03.079.

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